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Preparation of Virus-Enriched Inoculum for Oral Infection of Honey Bees (*Apis Mellifera*)

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Abstract

Honey bees are of great ecological and agricultural importance around the world but are also subject to a variety of pressures that negatively affect bee health, including exposure to viral pathogens. Such viruses can cause a wide variety of devastating effects and can often be challenging to study due to multiple factors that make it difficult to separate the effects of experimental treatments from preexisting background infection. Here we present a method to mass produce large quantities of virus particles along with a high throughput bioassay to test viral infection and effects. Necessitated by the current lack of a continuous, virus-free honey bee cell line, viral particles are amplified *in vivo* using honey bee pupae, which are extracted from the hive in large volumes using minimally stressful methodology. These virus particles can then be used in honey bee cage bioassays to test inocula viability, as well as various other virus infection dynamics, including interactions with nutrition, pesticides, and other pathogens. A major advantage of using such particles is that it greatly reduces the chances of introducing unknown variables in subsequent experimentation when compared to current alternatives, such as infection via infected bee hemolymph or homogenate, though care should still be taken when sourcing the bees, to minimize background virus contamination. The cage assays are not a substitute for large-scale, field-realistic experiments testing virus infection effects at a colony level, but instead function as a method to establish baseline viral responses that, in combination with the semi-pure virus particles, can serve as important tools to examine various dimensions of honey bee-virus physiological interactions.

Introduction

Honey bees (*Apis mellifera*) play a critical role in the modern global agricultural landscape but are currently suffering from a combination of biotic and abiotic stressors, including pesticide exposure, poor forage, parasites, and pathogens[1,2]. One

of the most important pathogens of concern are viruses, many of which are vectored by another of the major honey bee stressors, the parasitic Varroa mite (*Varroa destructor*). These viruses can cause an array of negative effects in honey bees including reduced brood survival, developmental defects, and paralysis that can lead to total hive collapse both before and after overwintering periods[3,4,5]. Although there have been promising advances in the development of technologies used to combat virus infection[6,7,8,9], the dynamics by which many viruses propagate, spread, and interact within a honey bee or colony are still poorly understood[5,10]. Understanding the basic biology of honey bee and virus interactions and their relationships with other environmental factors is critical for developing effective virus management techniques.

However, studying honey bee-virus interactions poses challenges with numerous known and unknown factors complicating the process. These include interactions with diet[11,12], pesticide exposure[13], and bee genetic background[14,15]. Even when focusing on virus infection alone, complications are common because honey bee populations, both managed and wild, always have some degree of background virus infection, though often without manifesting acute symptoms[16,17], and the effects of virus coinfection are not well understood[18]. This has made the study of honey bee virus effects difficult to disentangle.

Many honey bee virus studies have used circumstantial virus infections to look for interactions with other stressors, observing how background infections change with other treatments[12,19,20,21]. While this approach has been successful at identifying important effects, especially discovering how pesticide or dietary treatments affect virus levels and replication, inoculation with a virus treatment of known content and concentration is critical for experimental testing of virus infection dynamics. Even so, separating experimental treatment from background infection can also pose challenges. In field studies, researchers have differentiated strains of deformed wing virus (DWV) to provide evidence for virus transmission from honey bees to bumble bees[22], but using this approach would be difficult within honey bees alone. Virus infectious clones are a powerful tool, not just for tracking infection[23,24,25] but for reverse genetics studies of honey bee viruses and for virus-host interaction research[26,27,28]. However, in most instances, infectious clones are still required to fulfill the infection cycle inside cells to produce particles. Such particles are preferred as inocula for experimental treatments because their infectivity is higher than the naked viral RNA and inoculation with encapsidated genomes mimics a natural infection.

The production of pure, uncontaminated honey bee virus inocula (wild-type virus strains or those derived from infectious clones) also pose challenges. These are primarily due to the difficulties in obtaining a reliable, continuously-replicating, virus-free honey bee cell line to produce pure-strain viruses[29,30]. While some cell lines have been produced, these systems remain imperfect; still, there is hope a viable cell line can be produced[29], which would allow for finer control of virus production and investigation. Until such a line becomes widely available, most virus production protocols will continue to rely on the use of *in vivo* virus production and purification[18,31,32,33,34]. These approaches involve identifying and purifying virus particles of interest (or producing an infectious clone) and using them to infect honey bees, usually as pupae. The pupae are injected with the target virus and then sacrificed, and further particles are extracted and purified. However, because no bees are virus-free to begin with, there is always some degree

of contamination from traces of other viruses in any such concentrate, and, therefore, great care must be taken in choosing bees with a low likelihood of background infections. Further, methods for removing the pupae from the comb cells for use in these protocols[33] are very labor intensive and can induce stress in the bees, limiting production by these means[18,32]. Here, we report an alternative method that allows for large scale removal of larvae with little labor and less mechanical stress on the bees.

Once pupae are obtained and injected with the starting virus inoculum, they must be incubated to provide the virus time to replicate. Subsequently, produced virus particles can be processed into a form usable to infect experimental bees. There are several simple methods to achieve this, including using a crude homogenate[35,36] or hemolymph generated from virally infected bees as a source of infection[37]. These methods are effective but run into a greater chance of introducing unknown variables from the background substrate (e.g., other factors in the dead bee homogenates). Additionally, it is desirable to concentrate the particles if an experiment requires giving a large, known dose of a virus in a short period of time. Therefore, for better control, it is preferable to use methods that allow for some level of purification and concentration of the virus particles. Generally, a series of precipitation and centrifugation steps will result in the removal of almost all possible non-target virus material[33].

After producing this concentrated inoculum, it is beneficial to quantify the viral titers (qPCR) and characterize it with *in vivo* bioassays to test its viability and ability to cause mortality, as well as to corroborate that increased virus titers are obtained after infection. This can be achieved through injection experiments (either into pupae or adults) or feeding experiments (into larvae or adults). While all these approaches are possible, feeding to groups of adult bees in a cage is often the fastest and simplest. The cage assay method is also widely used for testing various other treatments on bees including pesticide toxicity[38], ovary development[39], and nutritional influence on behavior[40,41] and, therefore, can form a good basis for experiments linking virus infection with other factors[42].

Here we describe a reliable method for producing large quantities of semi-pure, highly-enriched virus particles without using an expensive ultracentrifuge, including a method for removing pupae that reduces labor and mechanical stress on the bees and a highly repeatable, high-throughput bioassay for testing viral infection and effects. By tightly controlling the purity of the viral inocula, investigators are able to reduce variation in honey bee viral response relative to other viral inoculation methods. Furthermore, the bioassay can screen for viral effects at a small groups level using highly repeatable experimental units before scaling to field-realistic settings, which is far more labor intensive to manage. In combination, these two methods provide the necessary tools for studies that can help improve our overall understanding of honey bee-virus physiological interactions.

Protocol

1. Mass bee extraction option 1: larval self-removal

1. Cage a honey bee queen on an empty, drawn-out Langstroth frame and return her to her colony. Allow the queen to lay eggs on this frame for 24 h.
    1. Check the frame after 24 h to ensure most of the comb cells contain newly laid eggs. Depending on the queen and colony, eggs are sometimes not laid

very well in the first 24 h. If this occurs, allow for an additional 24 h and adjust the time as necessary.

2. After the 24 h egg-laying period, release the queen. Mark the frame clearly and return it to the colony.

3. Exactly 192 h (8 days) after caging the queen (assuming normal egg laying within the first 24 h; 8 days marks the point right before pupation), remove the marked frame from the colony. Brush off all adult bees and transfer the frame to an incubator matching the internal conditions of a hive (34 °C and 50% relative humidity (RH)).

    1. Check to make sure most of the frame is filled with 5th instar larvae, which can be recognized by their large, white, c-shaped bodies pressed tightly against the bottom edges of the comb cells. There will likely also be a few cells already covered by a wax capping, especially near the center of the frame.

4. Prepare containers matching the height and width of the larvae-filled frame to receive the 5th instar larvae by thoroughly cleaning the inner and outer surfaces with soap and water, followed by a bleach solution, and finally ethanol. Thoroughly dry the container before proceeding.

    1. Line the bottom of the containers with several layers of paper towels. Then add several overlapping layers of thinner, absorbent cleaning wipes (e.g., delicate task wipers) or filter paper. The material must be absorbent. Avoid overlapping the top layer to improve the ease of future larval transfer.

5. Place frames face down (with focal larvae facing downwards) on top of the containers so that the larvae can fall onto the layer of cleaning wipes.

    1. Cover the container and frame with a tented piece of aluminum foil or other covering to retain moisture and return the setup to the incubator. Leave the setup overnight. The natural food-seeking tendencies of the larvae will cause them to crawl out of their cells and fall onto the padded surface below.

6. Prepare separate transfer trays by thoroughly cleaning them using the same steps detailed in 1.4. Allow trays to dry and layer the bottoms with cleaning wipes. The trays do not need to match any specific dimensions, but shallower trays will allow for easier larval manipulations.

    1. Begin transferring the larvae from the containers to the trays by carefully lifting off individual wipes from the top layer in the containers and gently pouring the larvae onto the trays. The larvae should have fallen into the containers overnight, forming several sticky masses (**Figure 1A**).

    2. Use blunt soft forceps to separate out the larvae and lay them out across the surface of the trays. They do not need to be evenly spaced and can be close to one another but should not be touching. See **Figure 1B** for a visual depiction of separated larvae.

    3. Take this opportunity to remove any damaged (discolored) or below-average size larvae. These are more likely to die during the maturation process and can bring infections/fungal growth throughout the tray.

7. Cover the tray with tented aluminum foil to retain moisture and return the setup to the incubator.

8. Check the larvae daily and remove any that are discolored (dark brown or black).

    NOTE: The larvae/pre-pupae will defecate onto the cleaning wipes, manifesting as small brown patches. They may also produce small quantities of white, wispy webbing as part of their capping process. Neither of these

occurrences necessitate the replacement of the cleaning wipes, as long as they are absorbent.

9. Allow larvae to pupate and mature to the white-eye stage, which can be identified by their general shape matching that of an adult bee while still lacking pigmentation in their eyes and most of the rest of their body. This should occur between 14 to 15 days after queen caging. The pupae are now ready for virus injection. **Figure 1C, 1D** for examples of white-eye pupae.

2. Mass bee extraction option 2: manual pupal excision

NOTE: Although option 2 (pupal excision) is a viable method of bee extraction, it also features several drawbacks when compared to option 1 (larval self-removal). Option 2 is far more labor intensive, harder to control for pupal age, and generally more stressful on the bees themselves. Option 1 is recommended whenever possible.

1. Select a frame of capped honey bee brood containing pupae at or near the white-eye stage (see 1.9 for a description) from a suitable colony. Remove the capping from comb cells located near the center and edges of the frame to confirm the presence of the appropriate developmental stage.
2. Transfer the frame to an incubator set to 34 °C and 50% relative humidity. Always return the frame to the incubator when not in immediate use.
3. Prepare and clean transfer trays identical to those described in 1.6.
4. Remove the frame from the incubator and set on an angled stand underneath a light source. Moisten a small stack of paper towels with water.
5. Clean a pair of blunt hard forceps using ethanol. Using the clean forceps, remove the capping from the cells containing white-eye pupae, taking care to not damage the pupae in the process.
6. One by one, gently excise the pupae from the comb cells. It is safest to grasp the pupae around the thorax and abdomen using the forceps tips, if there is sufficient space. If not, however, pupae can also be removed by grasping the head and slowly wiggling it out far enough to then be grasped around the body.
    1. Cover the parts of the frame that are not being immediately accessed with wet paper towels to retain moisture. Remove and replace as necessary during the excision process.
7. Space the excised pupae along the cleaning wipes in the transfer trays, making sure none of them are touching. Any disfigured or discolored pupae should be discarded. Pupae are now ready for virus injection.
    1. Discard pupae which release fluid upon contact with the wipes; they have likely been punctured. Sometimes, pupae will exhibit small dark spots of melanization near the points of contact with the forceps within 1 h upon removal. This should not affect survival. If large patches of melanization appear, the affected pupae should be discarded.

3. Pupal virus injection

NOTE: If performing this protocol for the first time (i.e., without prior viral inocula stocks), first extract and concentrate particles using adults, pupae, or larvae from a colony with a suspected infection. Measure the viral titers in the resultant inocula as described in step 5 and determine which particles to propagate further.

1. Sterilize all work surfaces using bleach water and ethanol before beginning work with honey bee viruses. Nitrile gloves should be worn during the entire process.

2. Prepare an injector apparatus capable of injecting fluid in ~1 µL amounts.

   NOTE: One inexpensive yet effective approach would be to create a handmade device by attaching a 30 G hypodermic needle to the tip of a 100 µL multi-dispenser tip (see **Table of Materials**) using flexible epoxy or another liquid adhesive. Make sure the edges of the needle cap are sealed tightly against the multi-dispenser tip and allow the apparatus to dry. See **Figure 2** for a visual depiction of an example injector apparatus.

3. Prepare a virus injection dilution solution by mixing the desired type of virus particles with sterilized 1x PBS (phosphate-buffered saline) in a 15 mL conical centrifuge tube. The total quantity needed will depend on the number of pupae that need to be injected, with each pupa requiring a 1 µL injection (e.g., 500 pupae = 5 µL virus particles + 495 µL PBS).

4. Attach the injector apparatus to a manual multi-pipette and test the efficacy by drawing up 100 µL of water from a separate beaker and dispensing it in 1 µL doses. Ensure that every amount dispensed is equal, swapping out injector apparatus as necessary. Clear any remaining water from the injector.

5. Retrieve the trays of pupae generated in step 1 or step 2 from the incubator and remove the aluminum foil covering.

6. Clean a pair of blunt hard forceps using ethanol. Gently grasp the individual pupa along the thorax, applying just enough pressure to force internal fluids to the abdomen. This should make the tergite divisions more apparent.

7. Draw up 100 µL of the virus particle solution using the multi pipette, insert the needle between the third and fourth abdominal tergites, and inject 1 µL of the virus solution. Repeat for every pupa laid out on the trays, taking care to avoid repeat injections. Any pupae damaged during the process should be discarded.

   CAUTION: All virus-preparation-related materials are designated as biohazards and should be autoclaved and disposed of following institutional guidelines. Needles should also be disposed of following institutional guidelines.

8. Cover the trays of injected pupae with aluminum foil and return to the incubator. Allow the virus particles to propagate within the pupae for 3-5 days.

   1. Perform daily inspections on the pupae and remove any dead or rotting specimens to prevent bacteria or fungal buildup. Small points of melanization may appear at the injection site on the abdomens but should not affect survival.

9. Sample the pupae into 50 mL conical centrifuge tubes and vortex to homogenize the contents, taking care to sample pupae into tubes by colony source to reduce contamination from non-target viruses. Ensure no whole pupae remain and transfer the tubes to a -80 °C freezer until ready for virus particle precipitation and concentration.

4. Virus particle concentration

NOTE: This protocol has not been tested for the recovery of enveloped viruses.

1. Autoclave all materials and containers before using. Nitrile gloves, lab coats, and eye protection should be worn during this entire process. Sterilize all working

surfaces using bleach water, ethanol, and RNase inactivating solution before beginning.

1. Prepare 1x TES (Tris-EDTA-salt) buffer: Mix 10 mM Tris-HCL pH 7.5, 2 mM EDTA, and 150 mM NaCl. Sterilize by autoclaving.

2. Thaw homogenized pupae and transfer to centrifuge bottles. Add approximately three volumes of 1x PBS (e.g., add a full 50 mL tube of pupae to 150 mL of 1x PBS) and equalize volumes to that of the fullest bottle.

3. Mix first by hand and then by placing onto an orbital shaker at room temperature for 10 min.

4. Centrifuge at 15,000 *x g* at 4 °C for 5 min to remove cellular debris. Repeat this step as needed if cellular debris remains.

    1. If the supernatant has large globules of fat floating at the surface, filter through cheesecloth into separate sterile centrifuge bottles before proceeding.

5. Extract the supernatant with 0.3 volumes of 24:1 chloroform:isoamyl alcohol solution (e.g., 190 mL of supernatant + 57 mL of chloroform:isoamyl alcohol) and mix by inversion. Centrifuge at 21,000 *x g* at 4 °C for 20 min.

    CAUTION: Avoid direct contact with chloroform on the skin or eyes. Always wear proper PPE. All chloroform waste should be disposed of following institutional guidelines.

6. Recover the aqueous phase from each bottle by decanting the supernatant into separate sterile 500 mL beakers in an ice bath or in a 4 °C cold room. Take care to avoid contaminating the supernatant with chloroform as it will make the purification process more difficult; it is better to lose a little bit of aqueous phase.

7. Add RNase free water to bring each beaker up to a volume of 200 mL. Place the beakers onto magnetic stir plates and drop in a medium-sized stir bar. Set the plates to stir at a medium-low setting.

8. Slowly add NaCl to each beaker under constant gentle stirring, bringing each up to a final concentration of 2.3% (e.g., 4.6 g NaCl per 200 mL of supernatant). Add polyethylene glycol 8000 (PEG) to each beaker up to a final concentration of 7% (e.g., 14 g PEG per 200 mL of the supernatant).

9. Cover the beakers with aluminum foil and continue to stir at a medium-low speed on ice or in a cold room for 1-5 h to dissolve the PEG. The more time spent stirring, the more thoroughly the PEG will dissolve.

10. Turn off the stir plates. Incubate the covered beakers for 1-3 days on ice or in a cold room to allow virus particles and proteins to precipitate. The more time spent incubating, the more particles that will precipitate.

11. Transfer the contents of each beaker into separate clean centrifuge bottles and centrifuge at 15,000 *x g* at 4 °C for 30 min to recover a PEG-particle pellet. Discard the supernatant.

12. Carefully scrape the PEG-particle pellet off the sides of the bottle and resuspend them in minimal volumes of 1x TES buffer inside clean beakers by slowly adding small quantities of TES to the pellets (approximately 10 mL per 100 original bees).

13. Pass the suspended pellet through an 18 G needle at least ten times before aliquoting into 2 mL centrifuge tubes. Keep all tubes on ice until ready for 4.14.

14. Centrifuge 2 mL tubes at 13,000 *x g* at 4 °C for 15 min to remove additional PEG. Transfer the supernatant into

another 2 mL centrifuge tube using a 1,000 µL pipette and repeat the centrifugation step to ensure complete removal of all PEG.

15. Concentrate the remaining particles within the supernatant into new centrifuge tubes using centrifugal filter units (100 kDa cutoff) via several rounds of centrifugation at 14,000 *x g* at room temperature (RT) for 10 min each until reaching approximately one fifth the original concentration (10 mL of particle-TES solution to about 2 mL of concentrated particles). The filter units come in different sizes; select the most appropriate for the number of samples being processed. The units that fits into 15 mL-sized conical tubes are usually the most appropriate.
16. Resuspend the concentrated particles by passing through a 26 G hypodermic needle and centrifuge at 14,000 *x g* at RT for 5 min for one final round of PEG removal. If the fluid is still cloudy, repeat until all PEG precipitate is removed.
17. Aliquot the viscous supernatant into desired quantities and store at -80 °C until ready for use.

5. Virus RNA extraction and quantification

1. Extract RNA from whole bees or concentrated virus particles using any appropriate RNA extraction method (e.g., TRIzol RNA extraction reagent followed by DNase treatment).
2. Quantify virus titers in inoculum generated in step 5.1 via RT-qPCR, preferably using a standard-curve based method that does not rely on host gene expression[18], though other methods may also allow for estimates.

6. Viral feeding bioassay

1. Prepare all necessary materials for the bioassay before the frame collection step (6.2) or during the 24-hr bee emergence period (6.3).
    1. Prepare clean cages or other enclosures capable of housing the number of bees necessary for the bioassay (e.g., acrylic box cages measuring 10.16 cm x 10.16 cm x 7.62 cm) by plugging all feeder holes with an appropriately sized centrifuge tube (**Figure 3**). Determine and randomize treatments among the cages and label each one with their designated treatment for easy future reference.
    2. Prepare inoculum trays by labeling individual medium-sized weigh boats with their corresponding cage and treatment.
    3. Prepare feeding solution by mixing the appropriate quantity of sucrose with deionized water (e.g., 300 g sucrose per 1 L water for a 30% sucrose solution), making sure to sterilize the water before and after adding sucrose. Sterile sucrose solution can be stored in a 4 °C refrigerator for several weeks but should be discarded if any cloudiness appears.
    4. Prepare feeder tubes by partially filling 15 mL centrifuge tubes with feeding solutions produced in 6.1.3, inverting them, and poking 1-2 holes around the tip of the tube with a thumbtack. Holes can also be melted using a 18G hypodermic needle heated over a flame. Make sure feeder tube caps are screwed on very tightly as loose-fitting caps can cause slow leaks that will drown cage inhabitants overnight.

        NOTE: Feeding solutions and feeder tube volume can be adjusted as necessary to suit experimental needs.

5. Prepare a collection receptable for newly emerged bees by lightly coating the edges of a large tub with vegetable oil or a similar greasy substance. Separately, prepare several small cups using the same coating method.

2. Select and remove frames of honey bee brood on the verge of eclosion sourced from at least three separate hives. Appropriately aged bees resemble fully pigmented adults underneath the comb cell capping. A sure sign of ongoing emergence is observing cell inhabitants slowly chewing their way out and/or recently emptied cells with characteristic jagged chew marks along the wax capping.
NOTE: The exact quantity of frames required will depend on the size of the experiment, the amount of emerging brood per frame, and the time of year. A standard Langstroth deep frame contains ~3,000 comb cells per side; a frame containing mostly capped pupae, with some observed emerging can easily produce 400+ bees in 24 h. Adjust throughout the season, as necessary.

3. Brush off all adult bees before placing the frames into emergence boxes and transferring them to an incubator matching the internal conditions of a hive (34 °C and 50% RH). Allow bees to emerge for 24 h.

4. Remove the emergence boxes from the incubator and brush all newly emerged bees into the collection tub. Make sure to remove all bees from the emergence boxes as well to prevent the inclusion of incorrectly aged bees in any subsequent brushings. Any bee able to fly emerged > 24 h ago and should be excluded from the bioassay.

5. Produce a homogenized mixture of newly emerged bees by gently mixing the bees in the collection tub by hand (they cannot sting at this age) to minimize the hive genetic effects from any individual colony. For specific applications, other arrangements for colony source may be desirable.

6. Count out 35 individual bees, placing each one into the same greased cup before transferring the contents to an acrylic cage. Alternatively, it may be easier to separate out smaller multiples of bees into several greased cups (e.g., 5 cups of 7 bees each) to avoid miscount errors. The number of bees used can be varied based on needs and applications.

7. Transfer the cages of bees to an incubator (34 °C and 50% RH), making sure to follow the randomized placement order created in 6.1.1 to minimize any potential microclimate effects.

8. Prepare workspace for virus work by cleaning all surfaces and pipettes with bleach water, ethanol, and RNase inactivating solution before beginning. Make sure to wear nitrile gloves whenever handling virus particles.

9. Prepare the virus inoculum of a desired concentration by thawing out an appropriate quantity of concentrated virus particles (step 4.17) and mixing thoroughly with sufficient sucrose solution (step 6.1.3) in a sterile container. For cages of 35 bees, each requires 600 µL of inoculum. Serial dilutions are recommended if the desired virus concentration is at or below 0.001%.

    1. For example, an experiment involving 40 cages that all need a 0.01% virus inoculum will require 24 mL of virus solution, which can be created by combining 240 µL of concentrated virus particles with 23.76 mL of sucrose solution. It is recommended to include approximately 15% overage in the initial volume as some losses are to be expected with viscous liquids.

10. Lay out the inoculum trays prepared in 6.1.2 sorted by treatment type and pipette 600 µL of the appropriate inoculum onto each tray.
    1. Carefully insert the inoculum trays into their corresponding cages, taking care to not accidentally release any bees. Take this opportunity to scan the bees and replace any that may have died in the transfer process.
11. Allow for complete consumption of the inocula (approximately 12-14 h) before removing the centrifuge tubes blocking the top feeder hole inserting the appropriate feeder tubes prepared in 6.1.4. This helps ensure that the bees in the cage share the inoculum evenly across the population. Sucrose solution is provided *ad libitum* and the tubes should be refilled as needed throughout the course of the experiment.
12. Record the mortality within each cage at 12 h intervals for the first 72 h of each experiment, following which shift the recording to 24 h intervals. Remove dead bees from cages to increase the ease of future counts by sliding the cage door up just far enough for a pair of forceps to reach in and scoop out dead bees. Make sure to sterilize the forceps over an alcohol lamp between cages to prevent viral cross-contamination.
13. Sample bees for viral titer measurements (5.1-5.2) by haphazardly selecting live specimens within each cage and placing them into centrifuge tubes on dry ice. Typically, three bees are sufficient to produce viral titer measurements at any given timepoint without also depopulating the cage.
14. Continue regular mortality measurements for as long as necessary.

Representative Results

Successfully following the protocols (**Figures 1**) for pupal injection and viral extraction should produce large quantities of virus particles. However, sampling and injecting pupae sourced from a variety of colonies at multiple time points maximizes the chances of acquiring target virus with low contamination. The dynamics by which viruses replicate and interact with one another within a honey bee is not well understood; coupled with the likelihood for preexisting infection, there is no guarantee that the injected (desired) virus will become the dominant in any given pupa, even if the pupae were sampled from the same original colony. **Figure 4** demonstrates the potential range of viral proportions that one could expect to see following extraction. The four displayed colonies represent a subset of a larger virus harvesting effort in which every pupa was initially injected with a ~95% Israeli acute paralysis virus (IAPV) inoculum. Although 10 out of the 16 colony samples involved in these extractions contained highly pure IAPV (> 95%), including some > 99% (e.g., Colony 1), other samples varied in their IAPV proportion (e.g., Colony 2-3), with some even being dominated by other viruses such as deformed wing virus (DWV) (e.g., Colony 4).

Table 1 provides additional context for the amplification level of the four viruses (BQCV, DWV, IAPV, SBV) shown in **Figure 4** in the form of RT-qPCR threshold cycle (C_t) values (the point at which a PCR target reaches the threshold of detection) and total virus genome equivalents (ge) per 100 ng RNA. C_t values can be used as a predictor of proportion, but ge values need to be calculated using a standard-curve based method[17] . Notice that the actual quantity of particles (i.e., ge) produced is dependent on the number of pupae processed and the filtering stringency during the extraction process.

Virus particle preparations (amplified inoculum) should be stored at -80 °C and it is recommended to aliquot them, as they will degrade significantly if subjected to multiple freeze-thaw cycles[18]. Additionally, dose-response assays should always be conducted prior to experimentation, as a multitude of external factors (hive genetics, bee health, etc.) can lead to highly variable viral response. Using data derived from experimental cages (**Figure 3**), **Figure 5** demonstrates such variability by comparing the dose-response survival curves of honey bees, fed the same IAPV particles during two different years. Despite identical testing parameters, including the same viral inocula and testing concentrations ranging from 0.01% to 0.0001% IAPV, the trials conducted in 2018 (**Figure 5A**) and 2019 (**Figure 5B**) produced noticeably different survival responses in all but the control treatment, which received no virus in its sucrose inoculum. Note that, if desired, LD_{50} calculations can also be performed at this point to obtain more precise mortality measurements[43], but this is usually not necessary as approximations are generally sufficient. In 2018, a 0.01% dose resulted in approximately 50% survival at 72 hours post-infection (hpi), thereby making it the default concentration for most viral cage experiments conducted that year. However, that same dose achieved near total mortality in 2019 at the same timepoint, and as a result, most viral cage experiments conducted that year received a 0.0001% IAPV inoculum instead. This significantly lower concentration reached the same levels of mortality as a 0.01% IAPV inoculum in 2018 while also using 100 times fewer virus particles.

These data were produced with bioassays using cages similar to the one diagrammed in **Figure 3**. The feeder holes in the top and side allow for easy diet control and the sliding cage door makes it simple to add or remove objects from the cage environment, such as inoculum trays or dead bees. However, the generalized viral assay protocol is not restricted to these types of cages nor diet choices and should be modified to suit experimental needs.

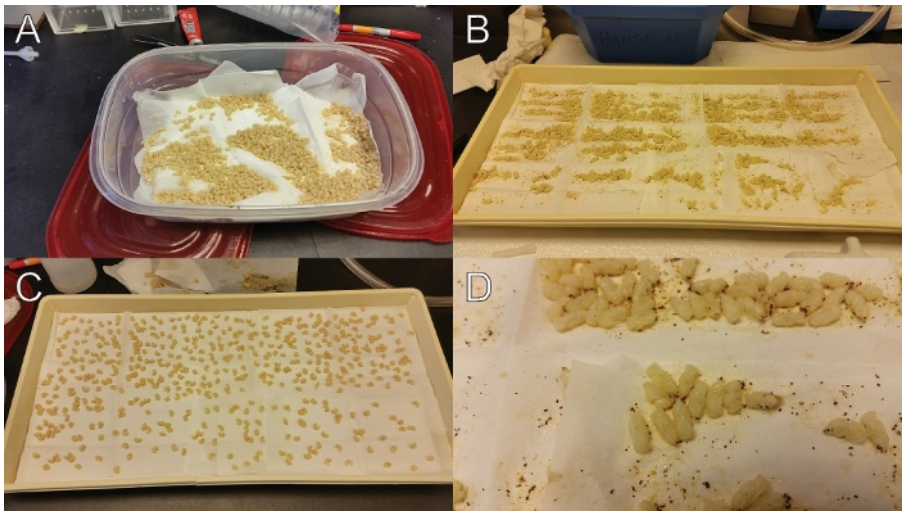

Figure 1: Representative images of various stages during the larval self-removal protocol. (**A**) Example larval mass that was expected following the overnight self-removal period (1.6). (**B**) Larvae spaced apart from one another on separate injection/growth trays (1.6.3) (**C,D**) White-eye pupae ready for viral injection (1.9). Brown spots are dried larval frass, which do not need to be removed. Please click here to view a larger version of this figure.

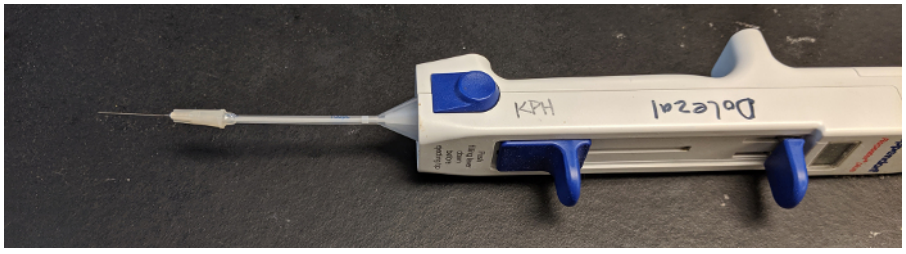

Figure 2: Example injector apparatus created by combining a hypodermic needle with a multi-dispenser tip. The needle and tip were joined using liquid adhesive to consistently deliver 1 µL fluid injections when attached to a repeating pipetter. Please click here to view a larger version of this figure.

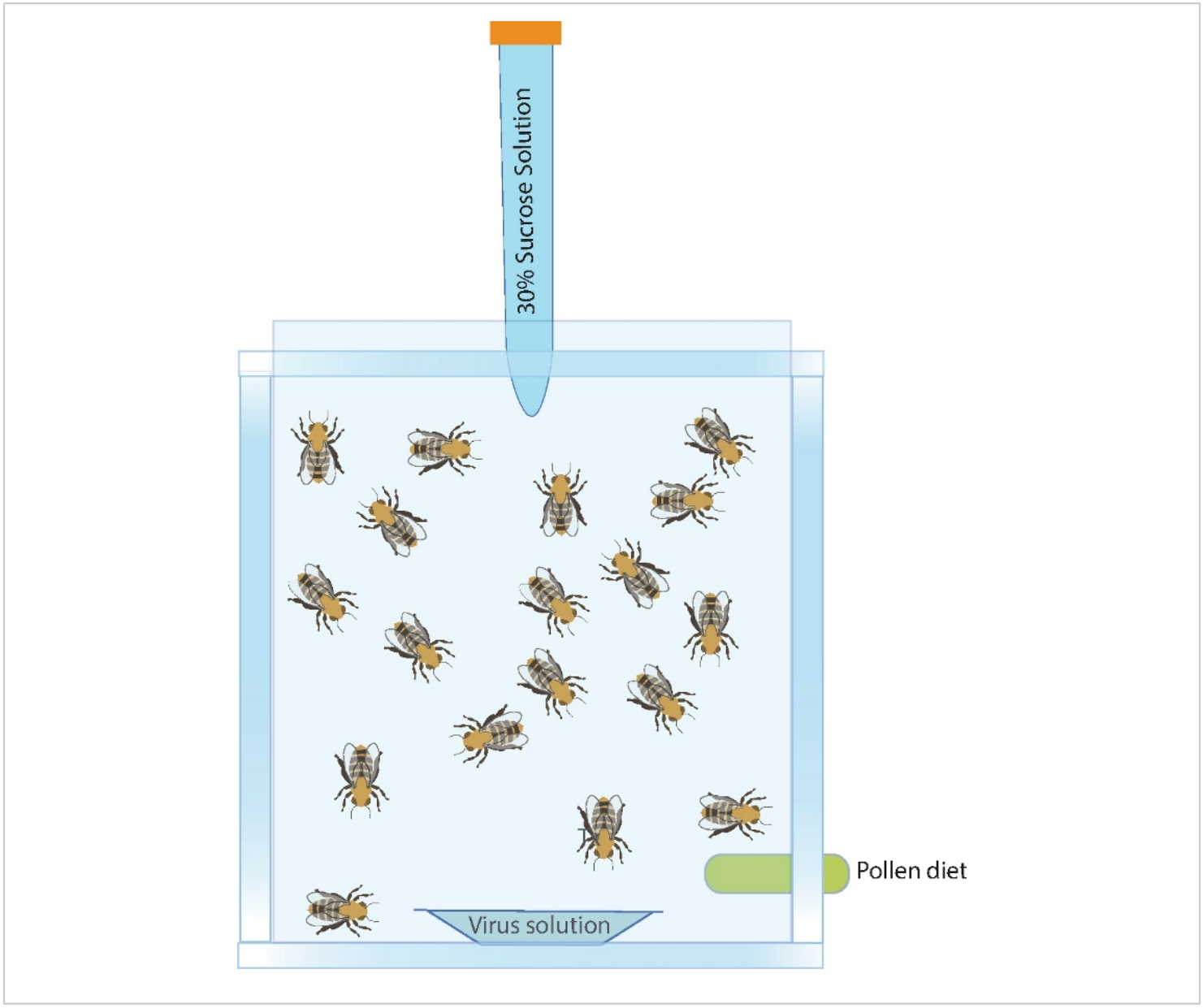

Figure 3: Example cage used in virus bioassay. Sucrose solution and pollen were provided *ad libitum* though feeder holes during the duration of the trial. Virus inocula could be easily delivered using a tray inserted through the bottom of the cage. Note that the cage type and feeder content could be adjusted as necessary to suit experimental parameters. Please click here to view a larger version of this figure.

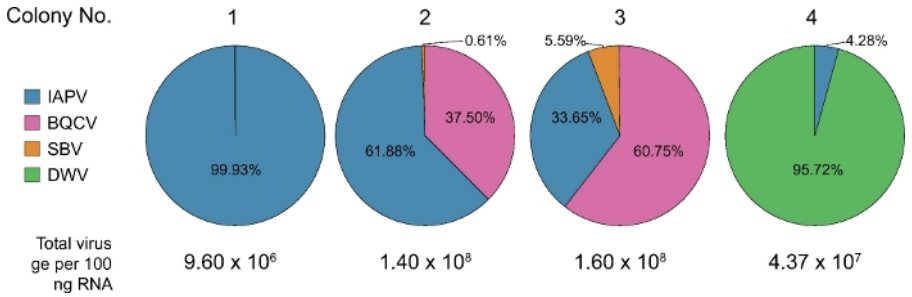

Figure 4: Average total virus loads and proportions across viral preparations from four sample colonies. Virus loads were measured by RT-qPCR as black queen cell virus (BQCV) + deformed wing virus (DWV) + Israeli acute paralysis virus (IAPV) + sacbrood virus (SBV) genome equivalents (ge) in 100 ng RNA. Each sample colony consisted of 150+ homogenized pupae originally injected with IAPV and represents the typical range of virus proportions generated from the pupal virus injection protocol. Please click here to view a larger version of this figure.

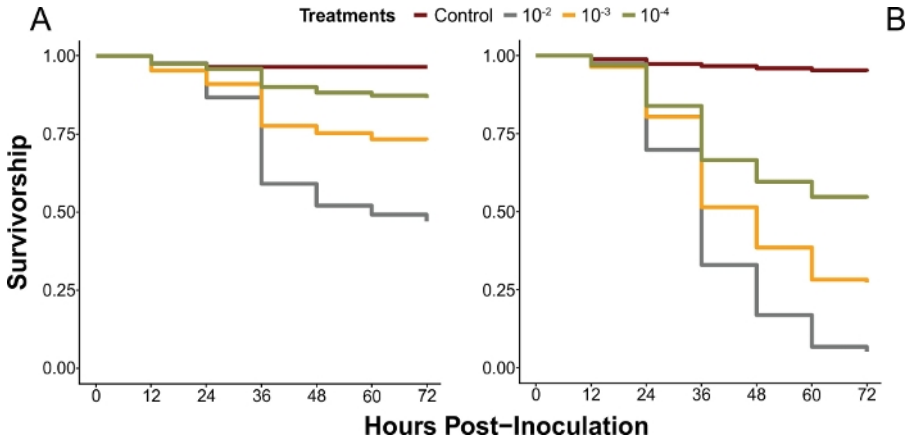

Figure 5: Dose-response survival curves of honey bee bioassay cages. Cages from both 2018 (**A**) and 2019 (**B**) were inoculated with IAPV and fed 30% sucrose solution *ad libitum* throughout the duration of the trial. Treatments represent IAPV inocula concentrations with 10^{-2} to 10^{-4} denoting 0.01% to 0.001% IAPV particle preparations mixed in 30% sucrose solution; control sucrose inoculations contained no virus. *n* = 56 total cages in 2018; *n* = 77 total cages in 2019. Please click here to view a larger version of this figure.

Table 1: Average threshold cycle (C_t) values and genome equivalents per 100 ng RNA of virus mixtures in four viral preparations from four sample colonies. Each sample colony consists of 150+ homogenized pupae generated from the pupal virus injection protocol. Viruses were detected by RT-qPCR using specific primers for each virus. Please click here to download this table.

Discussion

Here we have outlined methods detailing every step of the virus amplification and inoculum stock preparation process, including larvae collection and virus propagation, extraction, and concentration, as well as viral treatment in the form of cage-feeding experiments. These methods allow for production of semi-pure virus particles (**Figure 4**), the effectiveness of which can be consistently be quantified by dose-response mortality testing for viruses that are lethal to adults (**Figure 5**). Following confirmation of infective ability and/or pathology, the generated particles can then be used in bioassays to elucidate the interactions between honey bees and honey bee viruses.

One of the most distinctive benefits of the described protocols is that each is easily scaled to a large volume, whether it be pupae harvested, particles produced, or bioassays performed. Using pupal excision methods[33] , one can expect to remove ~100 pupae per hour, though this number will scale with proficiency. However, the larval self-removal method, reported here, while requiring different planning and scheduling procedures, can easily generate 10-20 times that number of removed bees overnight while involving comparatively little manual effort and less mechanical strain on the bees.

The main limitation of this method is that there is no way of guaranteeing that the self-removed larvae were not previously Varroa-infested. Regular mite treatment and monitoring of source hives can minimize this risk, but some larvae still may have had some level of Varroa parasitization. Manually removing pupae individually, however, allows the user to observe if any mites are present in the cell of a given pupa. Additionally, because the self-removal process relies on the food-seeking behavior of the larvae, the frames containing these larvae must be removed before the final feeding that normally occurs. Only due to this lack of provisioning by the workers do the larvae crawl from their cells. Therefore, the pupae derived from this method experience a very short window of nutritional stress compared to larvae that developed completely inside a colony and can appear slightly smaller. This is particularly notable in the youngest larvae in the cohort present on the frame; because the queen has laid eggs over a 24 h window, these larvae are missing proportionally more feeding time. These are usually clearly notable during pupation for their very small size compared to those removed by manual excision. However, the volume of larvae produced by self-removal more than compensates for their diminished individual biomass. Furthermore, the manual excision method also can cause substantial mechanical stress to the pupae as they are pulled from their cells. If either method is to be used as part of an experiment, and not just to produce virus particles, care should be taken to ensure proper controls.

Regardless of the extraction method, the virus propagation protocol can generate large quantities of virus particles using the pupae, which minimizes the variability induced by other currently practiced virus inoculation methods[35 , 36 , 37] when testing for honey bee viral response. It is important to note that this protocol was optimized and tested using non-enveloped viruses in the order *Picornavirales* (e.g., Israeli acute paralysis virus, deformed wing virus). Different strategies to isolate viral particles should be followed when working with enveloped viruses[44] . As a rough approximation, each of the 16 samples involved in the virus harvesting effort (which included the 4 colonies of **Figure 4**) were generated from 200-300 injected pupae and yielded between 2-2.5 mL of concentrated virus particle preparations. Assuming a virus inoculum concentration of 0.001% and a standard 35-

bee cage, each of the 16 virus preparations would provide sufficient particles for 3,300-4,200 cages. This surplus of infective material reduces experimental restrictions and enables high-throughput bioassays. It is important to note that although the virus particle concentrate can remain viable for months or years when stored at -80 °C, it can slowly decrease in infectivity, even if subjected to few freeze-thaw cycles. It is, therefore, recommended to store the viral preparation stock in small aliquots, several of which can then be used to quantify viral titers and verify the treatment dose at the time of the experiment. Additionally, the inter-year variability demonstrated in **Figure 5**, further reinforces the need for periodic dose-response testing, thereby minimizing the chances of unexpected loss in virus viability.

The cage bioassays themselves also have limitations, or at least caveats necessary to take into consideration, the primary one being the artificial nature of the cage bioassay environment (**Figure 3**). Grouping bees into enclosures allows for viral testing beyond the individual level; the cage becomes the experimental unit rather than the bee itself. Although this is more similar to an actual colony than a single bee being treated in isolation, it is still far from a realistic hive environment. Removed from the social environment, including queen and brood pheromones, bees of different ages, and other cues, these bees may not respond as a full-sized colony might. These are primarily considerations for larger-scale experiments using the cage system, however. The results gathered from cage viral bioassays should primarily be treated as baseline information establishment that can be used to inform future virus testing decisions scaled to a more field-realistic setting as desired by the user.

The methods described here provide a standardized process for the mass production of virus particles for use in honey bee viral assays. Such assays have already been implemented to examine various aspects of honey bee-virus interactions, including multi-virus infection and how diet quality and nutritional supplements affect survivorship in the face of viral infection[11,18,45,46]. They have been scaled up for use in colony-wide infection experiments[11,47] and to study the effects of infection on behavior[47] and gene expression[48]. Overall, these methods provide a baseline in tools that can be used to produce and evaluate honey bee virus inocula.

Disclosures

The authors have nothing to disclose.

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